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14. ABSTRACT
Mena, an actin regulatory protein, is upregulated in human breast cancer and is alternatively spliced to produce protein isoforms with distinct functions during tumor progression. The invasion-specific Mena^{INV} isoform, which is upregulated in invasive and motile cells, increases metastasis by potentiating tumor cell responses to EGF via dysregulation of the tyrosine phosphatase PTP1B. We show that expression of Mena^{INV} sensitized responses to other EGFR ligands as well as two other growth factors associated with tumor progression, HGF and IGF, whose receptors are known PTP1B substrates. Mena^{INV} can also drive invasion by affecting signalling downstream of integrins and via its direct interaction with α5β1. In 3D invasion assays, addition of fibronectin to a collagen gel drove invasion of Mena^{INV}-expressing cells in the absence of any growth factor ligand, an effect which was driven by its interaction with α5 and dependent on signalling via EGFR and Met. Furthermore, addition of fibronectin caused an even greater potentiation of EGF-induced invasion by Mena^{INV}, an effect which was dependent upon its interaction with α5. Overall, these results suggest that the pro-metastatic effects of Mena^{INV} involve its ability to bind α5 and regulate bidirectional signaling with FN and growth factors in the tumor microenvironment.

15. SUBJECT TERMS
metastasis, RTK signaling, integrins, MenaINV

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Introduction

Mena is a member of the Ena/VASP family proteins that regulate actin polymerization and modulate the morphology and dynamics of membrane protrusions thereby influencing cell motility (Gertler et al., 1996) (Bear and Gertler, 2009) (Bear et al., 2002) (Lambrechts, 2000) (Loureiro et al., 2002). Mena expression is upregulated in human breast cancers and plays a significant role in breast cancer metastasis (Gertler and Condeelis, 2011). Mena is alternatively spliced (Bear et al., 2000) and the Mena isoform containing the "INV" exon ("Mena^{INV}") is upregulated significantly in invasive cells (Goswami et al., 2009). Mena^{INV} increases tumor cell invasion, coordinated "streaming" motility, and tumor cell intravasation (Roussos et al., 2011a) (Roussos et al., 2011b) (Roussos et al., 2010) (Philippar et al., 2008). Mena^{INV}-expressing cells exhibit potent effects on EGF-elicited tumor cell motility, promoting actin polymerization and a more rapid and extensive lamellipodial protrusion, increasing invasion and sensitizing cells to respond to 50-fold lower EGF concentrations than control cells (Philippar et al., 2008). Analysis of several canonical signalling components downstream of EGFR (such as Erk and Akt) revealed no obvious effect of Mena^{INV} on their activities (Philippar et al., 2008) despite increased activation of the receptor itself (unpublished). The goal of this project is to further investigate the mechanism through which Mena^{INV} drives invasion as well as understand the dynamics of Mena^{INV} expression.

Body

Aim 1: Investigate the effects of Mena^{INV} expression on invasion promoting signaling

Expression of Mena^{INV} affects tumor cell response to EGFR ligands differentially. While Mena^{INV} sensitizes responses to the EGFR ligands EGF and HB-EGF, Mena^{INV} expression instead *dampens* responses to equivalent concentrations of TGFα (unpublished). These distinct responses may arise from the differences in receptor activation dynamics elicited by TGFα compared to other EGFR ligands (Ebner and Derynck, 1991) and indicate that inclusion of the INV exon could differentially regulate growth factor signaling. The goal of this aim is to investigate whether Mena^{INV} sensitizes cancer cells to signaling through other growth factor/receptor tyrosine kinase pathways relevant to breast cancer progression in both a membrane protrusion assay and in a 3D invasion assay.

Expression of Mena^{INV} sensitizes carcinoma cells to EGF, HGF and IGF

(Task 1a&b)

MDA-MB231 cells that have been engineered to express the different Mena isoforms were used to study the effect of stimulation with growth factors known to promote invasion. The following growth factors were tested: EGF, TGFα, HB-EGF, IGF, HGF, PDGF, NRG1, SDF-1, FGF-2, Amphiregulin (AREG). Cells were stimulated with the different growth factors at range of concentrations and the responses measured using the membrane protrusion assay and the 3D *in vitro* invasion assay. Work done in our lab compared responses to a panel of growth factors in several breast cancer triple negative cell lines in 3 assays: membrane protrusion, 2D motility and 3D invasion. Whereas 2D migration properties did not correlate well with 3D invasion, they found that increased membrane protrusion elicited by short term growth factor stimulation did relate robustly to enhanced 3D migration properties (Meyer et al., 2012). Therefore, both assays were used.

First, I confirmed that expression of Mena^{INV} in MDA-MB231 cells sensitizes cells to most EGFR ligands such as EGF, HB-EGF and Amphiregulin in both the protrusion and 3D *in vitro* invasion assay (Figure 1). Surprisingly, Mena^{INV} cells are unable to respond to TGFα in the 3D invasion assay, while control cells display a strong response to this growth factor.

Interestingly, Mena^{INV}-expressing cells were also sensitized to HGF shown here in the protrusion assay (Figure 2A). HGF did not elicit any motility response in the 3D invasion assay in any cell line at any concentration. The IGF sensitization was seen both in the protrusion and invasion assay (Figure 3). In the 3D invasion assay, there was no difference between the response of Mena and Mena^{INV} cells to either FGF-2, PDGF or SDF-1 in the 3D invasion assay (data not shown).

Interestingly, the receptors for HGF (Met) and IGF (IGFR) are known PTP1B substrates (Haj et al., 2003) (Ferrari et al., 2011). PDGFR is also a substrate for PTP1B, however we did not see sensitization to this ligand. Treatment with PDGF did not elicit a significant response in the 3D invasion assay or protrusion assay, which is likely due to the very low level of expression of this receptor compared to other RTKs in MDA-MB231 cells (Aaron Meyer, personal communication). Since previous work from the lab had shown that PTP1B dysregulation in the context of EGFR signaling was driving Mena^{INV} invasion, we decided to investigate whether Mena^{INV} could be causing dysregulation of several RTKs via PTP1B. The role of PTP1B in regulating Met activity has been well characterized and this occurs via phosphorylation of the twin Tyrosine residues 1234/5 (Sangwan et al., 2011). Here, we show that cells expressing Mena^{INV} show increased phosphorylation of Tyr1234/5 by Western Blot and immunofluorescence when stimulated with low doses of HGF (Figure 2 C&D). We have previously shown that Mena^{INV} cells are resistant to EGFR inhibitors Gefitinib and Erlotinib and show here that Mena^{INV} cells are also resistant to the Met inhibitor SU11074 in the protrusion assay (Figure 2E). Similarly, much higher doses of the Met inhibitor SU11074 are needed to shut down phosphorylation of Met in cells expressing Mena^{INV} cells (Figure 2F).

Investigation the signaling downstream of invasion-promoting growth factors
(Task 2)

A phosphoproteomic screen was recently completed in collaboration with the White lab here at MIT. Mass spectrometry was used to quantify tyrosine phosphorylation-mediated signaling networks downstream of EGFR. Human breast cancer cells (MDA-MB231) expressing Mena^{INV} or a control vector were starved and treated with several EGF concentrations (0, 0.25, 0.5 and 15.8 nM) and harvested at 1 min to generate a set of data relevant to the differences in EGF sensitivity caused by Mena^{INV} over the time course of the stimulation. 0.25 nM EGF evokes a protrusion response in Mena^{INV} cells but has no effect on control cells or cells expressing Mena (without INV). This analysis revealed a list of 35 proteins whose phosphorylation levels are significantly altered (>20%) when

Proteins involved in:	Proteins with increased pY in GFP-Mena^{INV} vs. GFP only cells
Integrin signaling	BCAR1, CAV1, EGFR, GRLF1, MAPK1, MAPK3, PXN, ROCK2, SHC1, PEAK1, PARD3
Regulation by PTP1B	ITSN2, CTTN, PXN, GAB1, ZO2, BCAR1, EGFR, TYK2, INPPL, GRLF1, Pragmin, GSK3, ARHGAP12, EphA2, SHB, CAV1, PKC
EGFR trafficking	ITSN2, INPPL, ANXA2, NWASP, CAV1, RIN1
Intracellular signaling at endosomes	ITSN2, Gab1, EGFR, SHC1, Ship2, NWASP, MAPK1, MAPK3
Interaction with Mena	INPPL, EGFR

Table 1: A subset of the hyperphosphorylated targets in Mena^{INV}-expressing cells as measured by quantitative phosphotyrosine-mass spectrometry after stimulation with 0.25 nM EGF for 60 sec.

Mena^{INV} is expressed after 0.25 nM EGF treatment.

The goal of this part of the project is to use the previously acquired mass spec data to identify and validate the signaling pathways who are most significantly regulated and involved in enabling Mena^{INV} -dependent metastasis. This screen has identified a subset of proteins that, due to the expression of the Mena^{INV} isoform, are significantly phosphorylated (up to 2.5 fold) within 1 min of EGF treatment, suggesting that these are the pathways through which Mena^{INV} exerts its effects on cell motility and could help explain how expression of this particular isoform makes cells so invasive. Further investigation into the function of the proteins identified in the screen reveal that in addition to regulating many PTP1B substrates, the second main group of substrates is related to integrin signaling. We found this to be very interesting in light of a recent paper published by our lab demonstrating that Mena can directly interact with α5β1 to regulate many of its functions, such as phosphorylation of Paxilin and FAK at focal adhesions, fibrillogenesis, spreading and cell motility (Gupton et al., 2012).

The idea that Mena^{INV} might be affecting signaling downstream of RTKs and integrins was extremely interesting to us. Indeed, integrins play an important role in integrating cells from the extracellular matrix and expression of both integrins and ECM molecules such as fibronectin and collagen are correlated with invasion and metastasis (Desgrosellier and Cheresh, 2010; Hynes, 2009; Lu et al., 2012) (Conklin et al., 2011). In addition, there is evidence linking integrin signaling and RTKs. Indeed, there is some evidence that certain ECM molecule can activate RTKs directly in the absence of any ligand: FN can activate Met without HGF present (Mitra et al., 2011) and Tenascin C with its EGF repeats can activate EGFR in the absence of ligand to activate downstream signaling pathways leading to motility (Iyer et al., 2008). In addition, there is evidence suggesting that RTKs can traffic with integrins to drive invasion. Mutant p53 expression can promote invasion, loss of directionality of

migration, and metastatic behavior through enhanced α5β1 and EGFR trafficking leading to constitutive activation of EGFR/integrin signaling (Muller et al., 2009). Therefore, we hypothesized that the interaction between Mena and α5β1 could be important for growth factor-mediated signaling in tumor progression.

Mena^{INV} and FN

In order to investigate the role of integrin signaling in the context of Mena^{INV} driven invasion, we added increasing amounts of FN to the collagen gels in the 3D invasion assay and examined how this affected basal invasion as well as responses to EGF (Figure 4).

First, we found that cells exhibited a biphasic dose response to increasing amounts of FN, where a dose of 50 µg/ml stimulated invasion without any growth factor present while a dose of 125 µg/ml no longer had that effect. The idea that cells might be refractory to high concentrations of ECM had previously been published (DiMilla et al., 1993) and this result in 3D is therefore not entirely surprising. However, we were surprised to find that cells expressing Mena^{INV} continued to invade at the high dose of FN and were not inhibited.

Secondly, the response of MDA-MB231 cells expressing Mena isoforms was also different, particularly when Mena^{INV} was expressed. Indeed, at 0.25nM EGF, addition of the low dose of FN (25 µg/ml) significantly increased the invasion response of Mena^{INV} cells compared to a condition without EGF or without FN (Figure 4C). These data demonstrate that both EGF and FN can drive invasion in the 3D in vitro assay in collagen gels. When cells express high levels of Mena^{INV}, these two signals can become additive, leading to increased invasion.

We started to investigate what could be driving the growth factor-independent FN-driven invasion in Mena^{INV} cells. Both Mena and Mena^{INV} interact with α5β1 in MDA-MB231 cells (Daisy Riquelme, personal communication). We hypothesized that the interaction between Mena^{INV} and α5β1 could be important in the response to FN in the 3D invasion assay. We observed FN-driven invasion in cells expressing Mena^{INV} with a deletion of the LERER region, which abrogates the interaction between Mena^{INV} and α5β1 (Gupton et al., 2012) (Figure 5A). In response to increasing amounts of FN, these cells are unable to invade compared to cells expressing Mena^{INV}. In addition, the effect of FN on Mena^{INV}-expressing cells was blocked by an antibody specifically blocking active α5 (P1D6) but not by cilengitide, a peptide inhibitor for αvβ3 (Muller et al., 2009) (Figure 5B&C).These data suggest that α5β1 and the interaction between α5β1 and Mena^{INV} plays an important role in FN-driven growth-factor independent invasion into a 3D collagen gel.

There is ample evidence in the literature supporting crosstalk between RTKs and integrins. We wanted to investigate if the FN-driven invasion involved signaling from RTKs, mainly EGFR and Met, whose ligands Mena^{INV} cells are sensitized to. Interestingly, blocking EGFR with Erlotinib or Met with SU110274 significantly decreased the FN-driven invasion in Mena^{INV}-expressing cells (Figure 5D&E).

Aim 2: Investigate the dynamics of INV inclusion

The second aim of this project is to develop a method to visualize the dynamics of INV inclusion during metastasis *in vivo*. Using a bichromatic reporter construct, we plan to study the role of the microenvironment on the inclusion of the INV exon, while also using it to study the function of newly identified signaling pathways from Aim 1.

Optimization and characterization of Mena^{INV} expression during tumor progression

To analyze Mena^{INV} protein expression and distribution, we developed an isoform-specific

monoclonal antibody that selectively recognizes the sequence encoded by the INV exon. The specificity of this antibody for the Mena^{INV} isoform in immunostaining assays was tested rigorously using Mena knockout mice, which are viable, and harbored an MMTV-PyMT transgene to generate Mena-protein null primary mammary tumors for use as negative controls for Mena^{INV} immunostaining (Figure 6). As additional controls, sections of xenograft tumors of MTLn3 cells expressing either ectopic GFP- Mena^{INV} or GFP-Mena were also stained. As expected, robust, specific anti-INV signals were detected in tumor tissue with wildtype Mena alleles or Xenograft tumors expressing Mena^{INV}, while the tumors from MMTV-PyMT Mena null animals and GFP-Mena-MTLn3 xenografts exhibited only trace signals, similar to the background observed when primary antibody was omitted from the protocol (unpublished observations, Shannon Hughes and Frank Gertler). Mena^{INV} levels were heterogenous throughout human and mouse. We noticed clusters of Mena^{INV}-positive cells throughout both mouse (Figure 6) and human (data not shown) tumors.

Since the epithelial-to-mesenchymal-transition (EMT) program is thought to endow tumor cells with many of the characteristics of aggressive, highly metastatic tumor cells (Valastyan et al., 2009), we hypothesized that expression of Mena^{INV} might be related to EMT progression within tumors. Therefore, we compared Mena^{INV} distribution to those of the cannonical EMT markers E-cadherin (epithelial) and Vimentin (mesenchymal) by immunostaining sections of mouse and human tumors. Comparison of Mena^{INV} and Vimentin distributions indicated within the entire population of Mena^{INV} -expressing cells, 62 and 74% also expressed detectable Vimentin in human and mouse tumors, respectively (Figure 7A &B). Conversely, only 27 and 18% of Vimentin-positive cells in human and mouse tumors, respectively, also expressed detectable Mena^{INV} (Figure 7A &B). We next examined the expression of Mena^{INV} and Ecadherin in PyMT mouse tumor sections. The relative staining intensities along line scans of images appeared to show that areas with higher Mena^{INV} levels generally had lower E-cadherin levels (Figure 7C). Quantitative analysis of the Mena^{INV} and E-cadherin immunofluorescence throughout entire microscopic fields within tumors revealed a significant, inverse correlation between relative levels of Mena^{INV} within the fraction of the tumor cell population containing the highest expression of E-Cadherin, and vice-versa (7 D&E). Together these results indicate that, while an EMT is not necessary or sufficient for Mena^{INV} expression in tumors, Mena^{INV} -expressing cells frequently exhibit characteristic of cells undergoing, or that have undergone an EMT as reduced E-cadherin expression and increased Vimentin levels, both hallmarks of passage through EMT, frequently accompany Mena^{INV} expression in tumor cells.

Mena^{INV} expression in cultured cells

In order to accurately validate the INV reporter that was generated, it was important to find a culture cell line that expressed high levels of both Mena and Mena^{INV}. When considering an appropriate cell culture model of human breast cancer to study the regulation of Mena^{INV} expression, we initially searched publicly available RNAseq datasets to identify human breast cancer cell lines that exhibited robust levels of Mena mRNA containing the INV exon (data not shown) but failed to identify any human cancer cell lines containing more than trace levels of INV exon inclusion compared to total Mena mRNA (1-2%; unpublished observations, Shannon Hughes & Frank Gertler). Western blot analysis using the anti-INV antibody indicated that Mena^{INV} protein was undetectable in 20µg of whole cell lysates prepared from several commonly used breast cancer cell lines (BT549, MCF7, MDA-MB 453 and SKBr3). After substantial enrichment by immnuprecipitation of 15mg of protein with the Mena antibody, Mena^{INV} protein was detectable in Mena IPs from triple negative (MDA-MB 231, BT549) cell lines, but was barely detectable in ER+/PR+ (MCF7, MDA-MB 453) and HER2+ (SKBr3) cells (Shannon Hughes, personal communication).

Since, Mena^{INV} mRNA is highly upregulated in invasive tumor cells *in vivo* (Goswami et al., 2009), and our immunostaining data revealed Mena^{INV} protein expression in mouse mammary, and human breast carcinomas, we measured Mena^{INV} protein levels by quantitative ELISA using protein lysates prepared from human breast cancer cells (MDA-MB231) in culture or from tissue isolated from

xenograft tumors generated by implanting either MDA-MB231 cells into the mammary fat pads of nod/scid mice (Figure 8). Tumor tissue derived from implanted MDA-MB 231 cells contained significantly more Mena^{INV} (174 pg Mena^{INV}/µg total protein or roughly 0.02% of total protein) than cultured MDA-MB 231 cells in which only trace amounts (if any) of Mena^{INV} protein could be detected by the ELISA assay. These data demonstrate that the tumor microenvironment is required for robust expression of the Mena^{INV} protein by tumor cells. Therefore, these data confirm that while MDA-MB231 have the ability to switch on Mena^{INV} in vivo with the right microenvironmental cues, they cannot be used to study in vitro expression of Mena^{INV}.

The only other organ in the body when Mena^{INV} expression has been detected is the developing nervous system. Therefore, we cultured cortical neurons dissected from an E18 embryonic mouse and looked at the expression of Mena^{INV} using the anti-INV antibody. While most cortical neurons expressed Mena, only a subpopulation of neurons expressed Mena^{INV} (Figure 9). Nevertheless, we proceeded with this cell culture system for further validation as this was the only cell type where we could detect Mena^{INV} by immunostaining.

Generation and validation the Mena^{INV} bichromatic reporter
(Task 5 a & b)

The reporter we generated expressed YFP and TdTomato in a mutually exclusive manner, depending on the splicing of the alternative exon cassette. The splicing of Mena can then be monitored as the ratio of the YFP to TdTomato fluorescence intensities. My experience with neuronal cultures as well as the fact that several members of the lab are currently working on neurons will make this step very easy. We nucleofected the construct as well as an empty vector control into neurons (Figure 10). Unfortunately, we were unable to get high levels of nucleofection efficiency in these cells. This can happen with neurons, and sometimes one needs to use a neuron-specific promoter to drive higher levels of expression. A very small number of neurons did express the reporters and signal in the red channel from the TdTomato was much lower in the empty control vector (Figure 10A) than with the INV reporter (Figure 10B). This would suggest that the reporter could be working. However, more work needs to be done to validate this reporter further.

Future plans: Using cells in culture does not seem like a viable option. We have decided to continue the validation in vivo, where we see robust levels of Mena^{INV} expression by ELISA. We will stably express the reporter in MDA-MB-231 cells and then inject cells into the mammary fat pad of mice and wait for tumors to form.

Key research accomplishments

- Evaluation of sensitization of Mena^{INV} expressing MDA-MB-231 cells to various growth factors in the protrusion and 3D invasion assay (Task 1 a & b)
- Uncovered Mena^{INV} affects signaling downstream of various RTKs (mainly Met and IGFR in addition to known EGFR) via dysregulation of a common phosphatase PTP1B
- Mena^{INV} drives resistance to clinically used Met inhibitor via increased phosphorylation of the receptor
- FN drives invasion of Mena^{INV}-expressing cells in the absence of growth factor, an effect which is dependent on Mena^{INV}'s interaction with α5β1 as well as EGFR and Met.
- Presence of FN in a 3D collagen gel sensitizes Mena^{INV}-expressing cells to EGF. These data suggest cross talk between RTKs and integrins, whose signal is amplified by expression of Mena^{INV}.
- Generation and validation of the INV reporter *in vitro* (Task 5 a & b)
- Optimization of staining for endogenous Mena^{INV} in mouse and human tumor sections and characterization of Mena^{INV} expression in human and mouse tumors

Reportable outcomes

1. Manuscripts

Hughes-Alford SK*, **Oudin MJ***, Neill J, Tadros J, Lussiez A, Riquelme D, Wyckoff J, White F, Condeelis J, Lauffenburger D, Gertler FB (2013) Phosphatase dysregulation underlies Mena^{INV}-mediated tumor cell invasion, Cell, in preparation to be submitted Dec 2013

2. Presentations
   a. Poster AACR Tumor Invasion and Metastasis Workshop, Jan 2013 (poster) "Mena^{INV}-mediated dysregulation of receptor tyrosine kinase signaling"
   b. Poster Ludwig MIT Retreat, May 2013 (poster) "Mena^{INV}-mediated dysregulation of receptor tyrosine kinase signaling"
   c. Poster Integrative Cancer Biology Program PI Annual Meeting, May 2013 (poster) "Phosphatase dysregulation underlies Mena^{INV}-driven invasion"
   d. Poster Integrative Cancer Biology Program, MIT Annual Retreat, July 2013 (poster) "Mena^{INV}-driven metastasis: a role for the extracellular matrix?"
   e. Poster Koch Institute Annual Retreat, October 2013 (poster) "Mena^{INV}-driven metastasis: a role for the extracellular matrix?"
   f. Poster NCIO-Nature conference 'Frontiers in tumor heterogeneity and plasticity', October 2013, "Mena^{INV}-driven metastasis: a role for the extracellular matrix?"
   g. Oral presentation, Wolfson Center for Age-Related Diseases, King's College London, October 2013 "Mena from Brains to Cancer"
   h. Oral presentation, Koch Institute Friday Focus Seminar Series, November 2013 'Sticky fingers: feeling your way through the matrix'

3. Conferences and workshops

Jan 2013: AACR Tumor Invasion and Metastasis Workshop, San Diego California, USA

May 2013: Integrative Cancer Biology Program PI Annual Meeting, Rockville, MD

May 2013: Annual Ludwig Institute Retreat Harvard and MIT, Boston, MA

July 2013: Annual Integrative Cancer Biology Program Retreat, Boston, MA

October 2013: Nature-CNIO Cancer Symposium ‘Frontiers in Tumor Heterogeneity and Plasticity’, Madrid, Spain

November 2013: AACR Workshop ‘Translational Cancer Research for Basic Scientists’
Selected to attend workshop

November 2013: MIT Office for the Dean of Graduate Education ‘Path of Professorship’ Workshop, MIT, Cambridge, MA

Conclusions

The first year of this fellowship has been very successful and I have reached all the objectives I had set for myself before starting. I established that Mena^{INV} can drive invasion by regulating several RTKs, mainly EGFR, Met and IGFR through dysregulation of PTP1B. Mena^{INV} does not affect responses to growth factors whose receptors are not regulated by PTP1B. Mena^{INV} also drives resistance to several RTK inhibitors, mainly Erlotonib for EGFR and SU110274 for Met. This is a clinically relevant finding, in light of the fact that RTK inhibitors are currently being used and in clinical trials for the treatments of various cancers. The main problem with using RTK inhibitors in the clinic is that patients develop resistance to them. Evaluating expression levels of Mena^{INV} expression in a tumor biopsy could be used as a predictive marker to identify patients who are most likely to develop resistance to these drugs.

In trying to better understand other ways that Mena^{INV} can drive invasion, we focused on a potential role for Mena^{INV} in integrating signals from the ECM via integrins due the fact that a large proportion of targets with altered phosphorylation identified by mass spec were known to be involved in integrin signaling and that we recently found that Mena interacts with α5β1 and that this interaction is important signaling downstream of integrins (Gupton et al., 2012). The data collected suggest cross talk between RTKs and integrins, with expression of Mena^{INV} amplifying signals downstream from both. These data point to the importance of Mena^{INV} in integrating signals from two signals known to drive invasion and metastasis. The pro-invasive effect of MenaINV further suggest that better understanding its mechanism of action could be important for the development of new targeted therapies in the treatment of metastatic disease. We are further investigating the role of Mena^{INV} and its interaction with α5β1 in the presence of growth factor signaling.

The second aim of this project is to study the dynamics of Mena^{INV} expression and INV inclusion and significant progress was made in the last year with regards to this aim. While the anti-INV antibody had been generated and optimized for use in cell lines in western blotting and immunofluorescence in over expressing cell lines. I developed a protocol to detect endogenous Mena^{INV} in both mouse and human tumors and characterized expression of Mena^{INV} in mouse and human tumors. I cloned the INV reporter and expressed in various cell lines. However, due to the very low expression levels of Mena^{INV} in cultured cells, cancer or neuronal, it has now become necessary to express this reporter stably in cell lines to make tumors in mice. We have shown that Mena^{INV} expression is significantly upregulated in MDA-MB231 cells injected in a mouse fat pad compared to MDA-MB231 cells in culture. Therefore, we are now planning to generate tumors from cells stably expressing the reporter to validate it and study the dynamics of INV inclusion and expression.

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Supporting data (ALL UNPUBLISHED DATA)

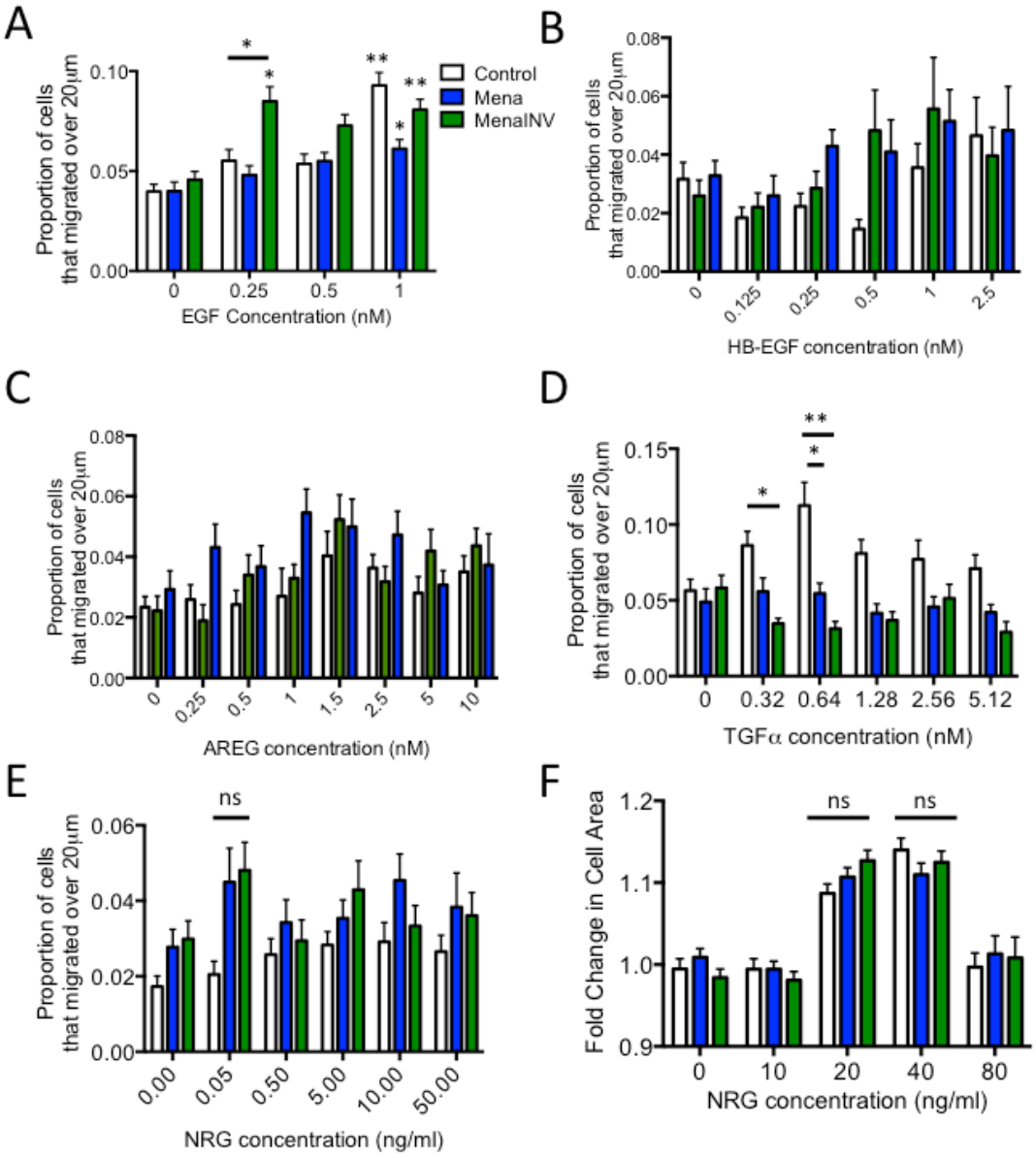

Figure 1: Mena^{INV} expression affects responses to certain ErbB ligands
MDA-MB231 cells expressing GFP-Control, Mena and Mena^{INV} were plated in a 3D collagen gel and treated with various growth factors which activate ErbB receptors EGF (A), HB-EGF (B), AREG (C), TGFα (D) and NRG (E). MDA-MB231 cells expressing GFP-Control, Mena and Mena^{INV} were also plated on collagen and matrigel, stimulated with NRG for 8 mins (E). Mena^{INV} expression sensitizes cells to EGF, with similar trends seen for HB-EGF and AREG, dampens responses to TGFα and has no effect on responses to NRG.

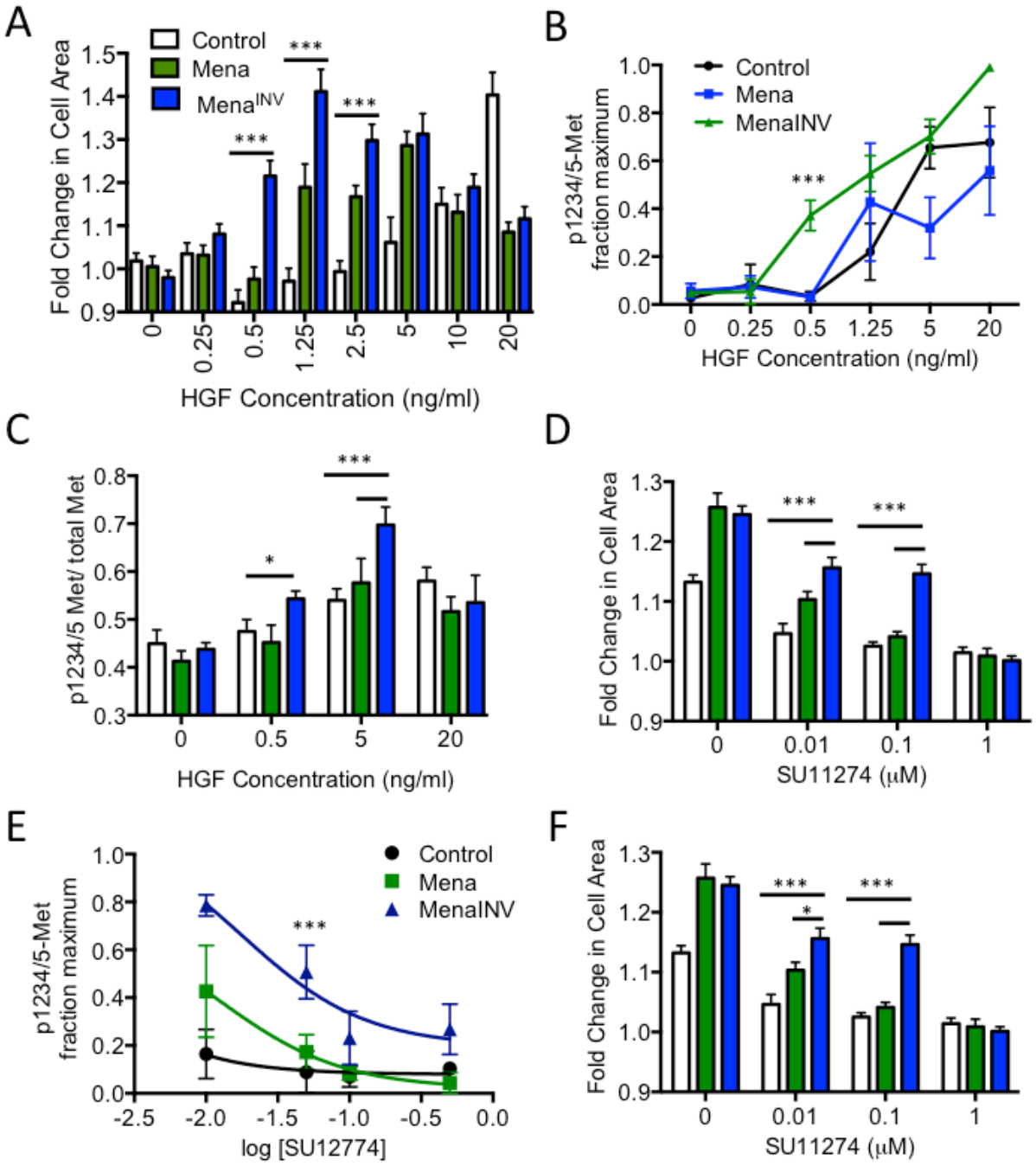

Figure 2: Mena^{INV} sensitization to HGF

MDA-MB231 cells expressing Mena^{INV} required less HGF to protrude (A) then cells expressing Control-GFP or Mena. Stimulation with low doses of HGF for 3 mins lead to increased phosphorylation of Tyr1234/5 on Met in Mena^{INV} cells compared to Control-GFP or Mena cells as shown by Western Blot (B) and immunofluorescence (C). MDA-MB231 cells expressing Mena^{INV} required more Met inhibitor SU12774 to inhibit the protrusion response to 5 ng/ml HGF (D). Stimulation with 5 ng/ml of HGF for 3 mins in the presence of increasing concentrations of the Met inhibitor SU12774 leads to increased phosphorylation of Tyr1234/5 on Met in Mena^{INV} cells compared to Control-GFP or Mena cells as shown by Western Blot (E) and immunofluorescence (F).

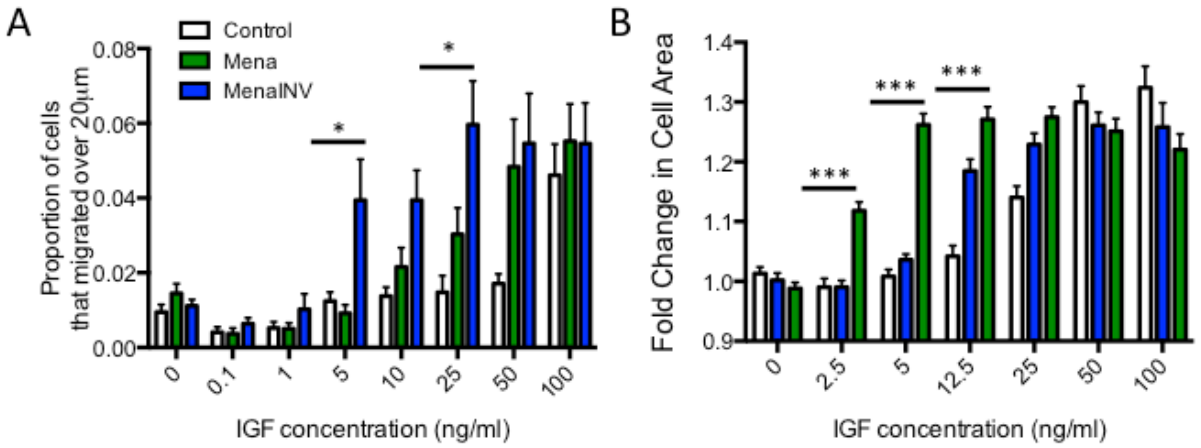

Figure 3: Mena^{INV} sensitization to other PTP1B targets
MDA-MB231 cells expressing Mena^{INV} required less IGF to invade in a 3D collagen gel (A) or protrude (B) compared to control-GFP and Mena-expressing cells.

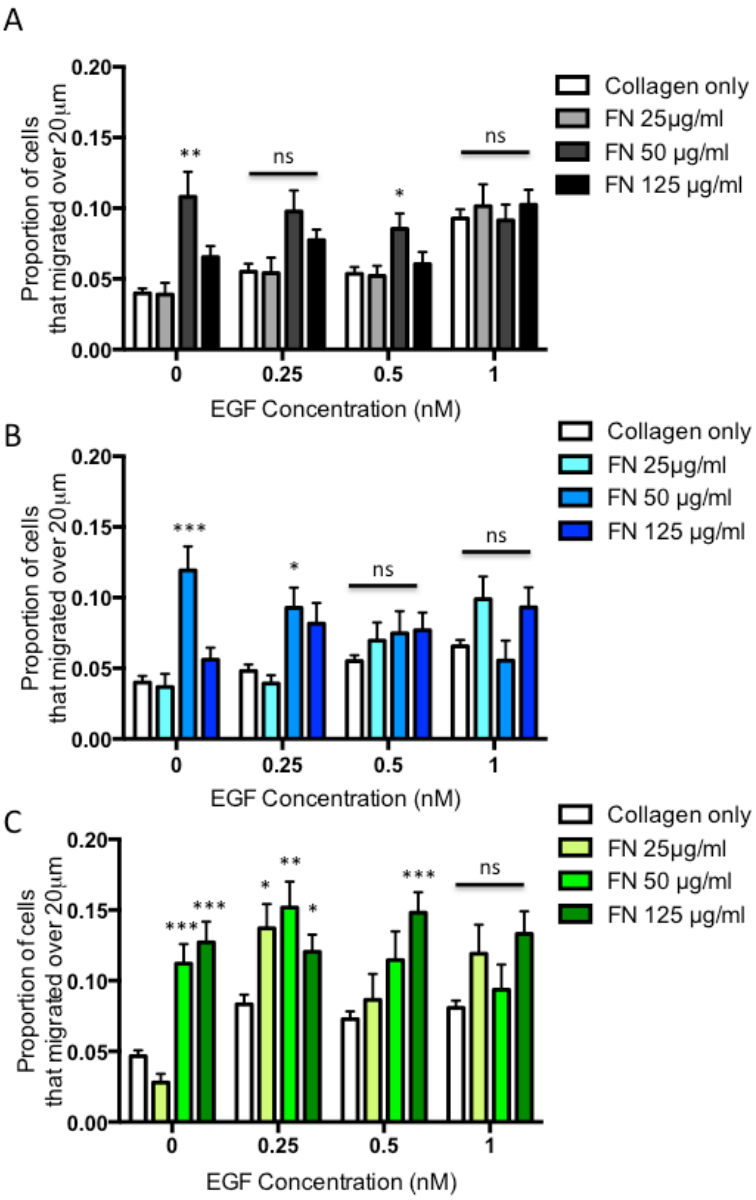

Figure 4: FN affects baseline invasion and response to EGF in 3D invasion assay
MDA-MB231 cells expressing GFP-Control (A) Mena (B) and Mena^{INV} (C) were plated in a 3D collagen gel with increasing amounts of FN (25, 50 or 100 µg/ml) and treated with various EGF concentrations for 24h. FN drives invasion without any EGF present and also affects responses to EGF.

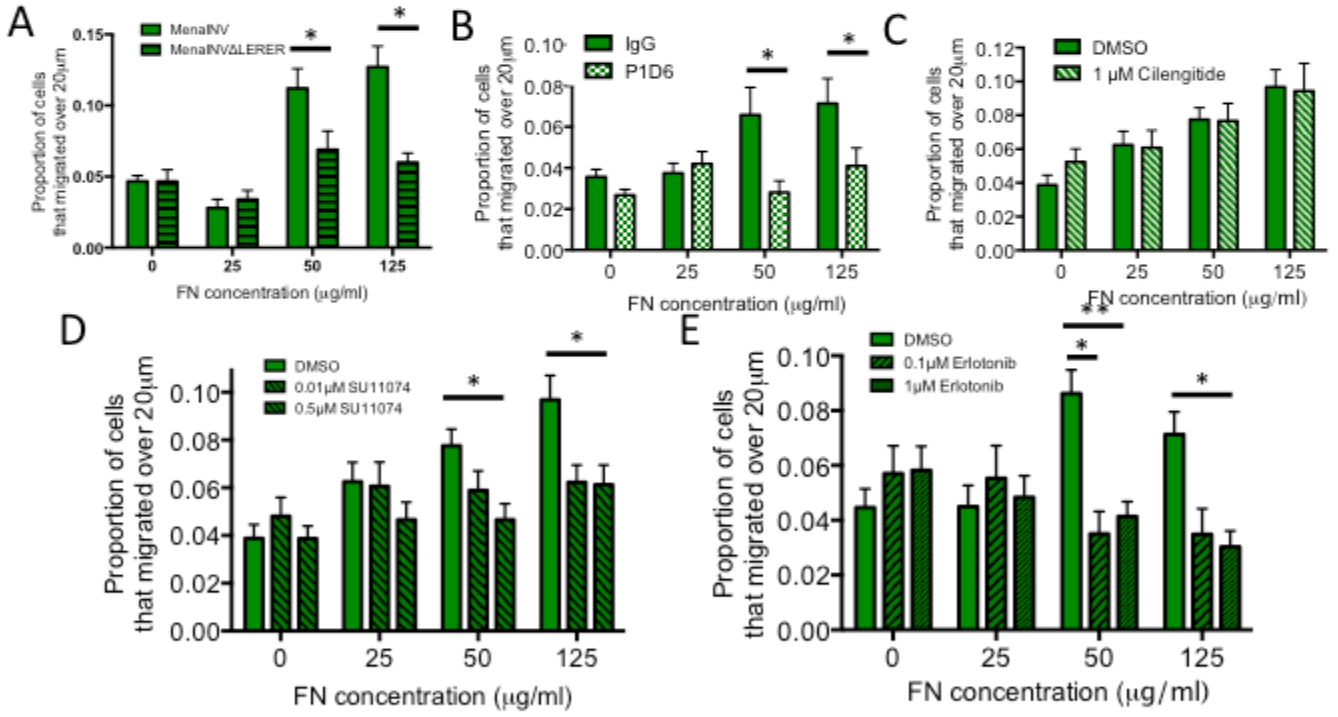

Figure 5: Growth factor-independent FN-driven invasion is mediated by α5β1 and RTKs
(A) MDA-MB231 cells expressing Mena^{INV} ΔLERER were plated in a 3D collagen gel with increasing amounts of FN (25, 50 or 100 µg/ml). MDA-MB231 cells expressing Mena^{INV} were plated in a 3D collagen gel with increasing amounts of FN (25, 50 or 100 µg/ml) in the presence of (B) 0.5 µg/ml P1D6, active α5 blocking antibody, (C) 1 µM cilengitide, peptide inhibitor of αvβ3, (D) 0.01 or 0.5 µM SU110274 Met inhibitor and (E) 0.1 or 1 µM Erlotonib EGFR inhibitor.

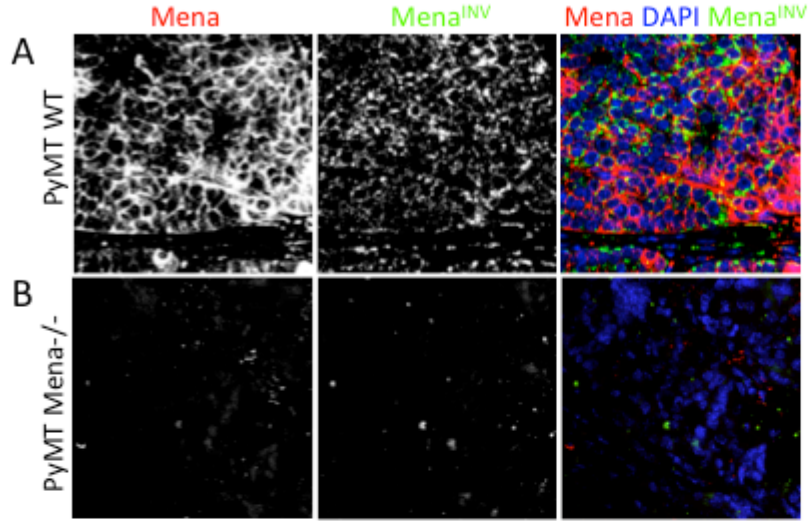

Figure 6: Endogenous Mena^{INV} in PyMT mouse tumors
MMTV-PyMT tissue section from a WT mouse (A) and a Mena -/- (B) mouse were stained for Mena and Mena^{INV} confirming the specificity of both Mena and Mena^{INV} antibodies. Nuclei stained with DAPI. Scale bar = 20 ìm.

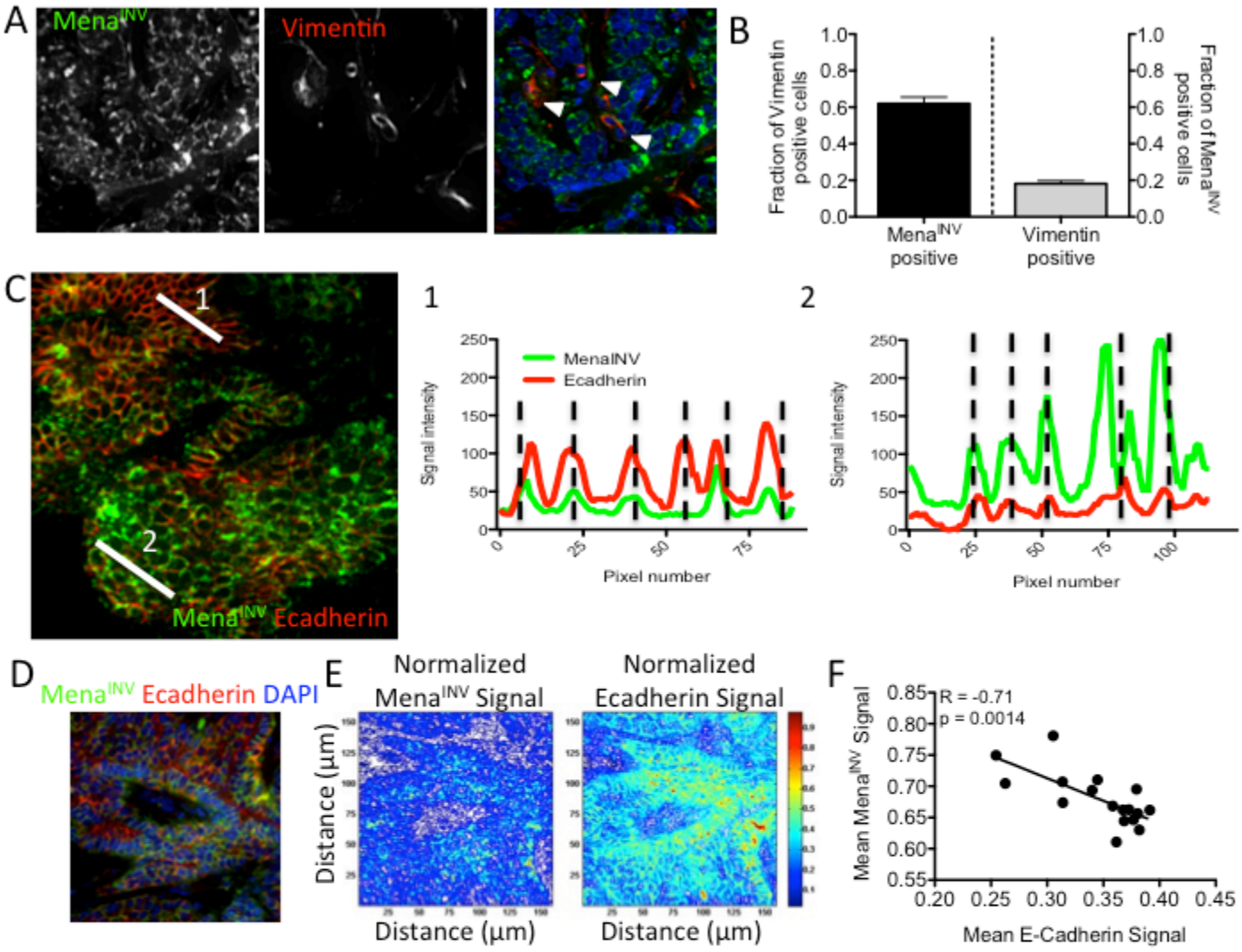

Figure 7: Mena^{INV} expression in breast cancer tumors is inversely correlated with E-Cadherin
(A) MMTV-PyMT tissue section stained for Mena^{INV} (red) and Vimentin (green). Arrowheads illustrates cells expressing both proteins. Scale bar = 20 ìm. (B) Black bar quantifies the fraction of Vimentin-positive cells that are also Mena^{INV}-positive, grey bar quantifies the fraction of Mena^{INV}-positive cells that are also Vimentin-positive. Results are shown as mean +/- standard deviation, n = 2 mice, 5-7 image fields per tumor. (C) MMTV-PyMT tissue section stained for Mena^{INV} (green) and E-Cadherin (red). (C1-D2) Pixel intensity values representing Mena^{INV} (green) and E-Cadherin (red) for the lines numbered 1-2 in (C). (E) Pixel intensity for normalized Mena^{INV} and Ecadherin signals from imaged in (D). (F) Inverse correlation between relative levels of Mena^{INV} within the fraction of the tumor cell population containing the lowest expression of E-Cadherin.

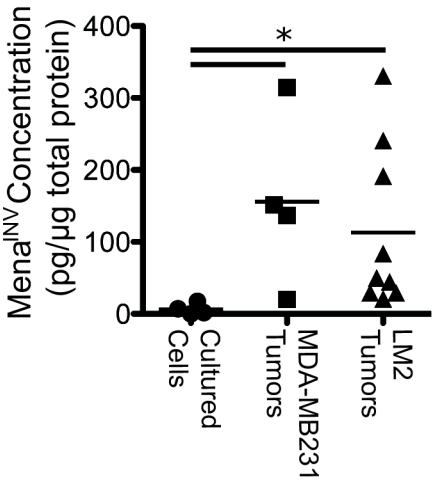

Figure 8: Mena^{INV} expression is microenvironment-dependent
Mena^{INV} protein concentration in plated versus mammary fat pad-implanted MDA-MB231 and LM2 human breast cancer cells quantified by sandwich ELISA using anti-pan-Mena for capture and anti-Mena^{INV} for detection. Asterisk indicates statistically significant difference by t-test (*p< 0.05).

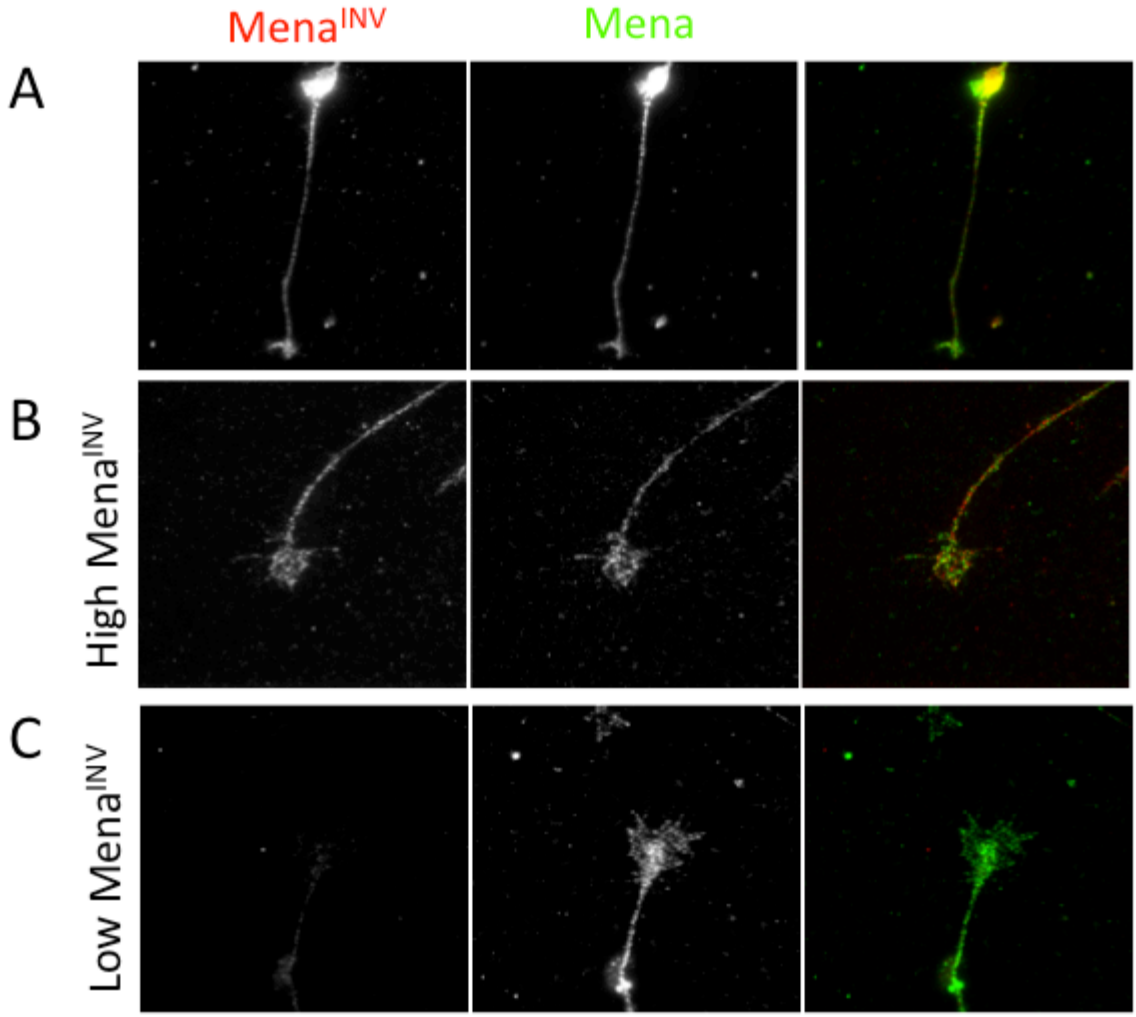

Figure 9: Mena^{INV} expression in cortical neurons
Cortical neurons dissected from E18 mouse brains were dissociated and plated on laminin and poly-Lysine and left for 2 days in culture. Immunostaining for Mena (green) and Mena^{INV} (red) shows expressing in both axons and cell bodies (A), with some neurons showing high Mena^{INV} expression (B) and some expressing very little Mena^{INV} (C).

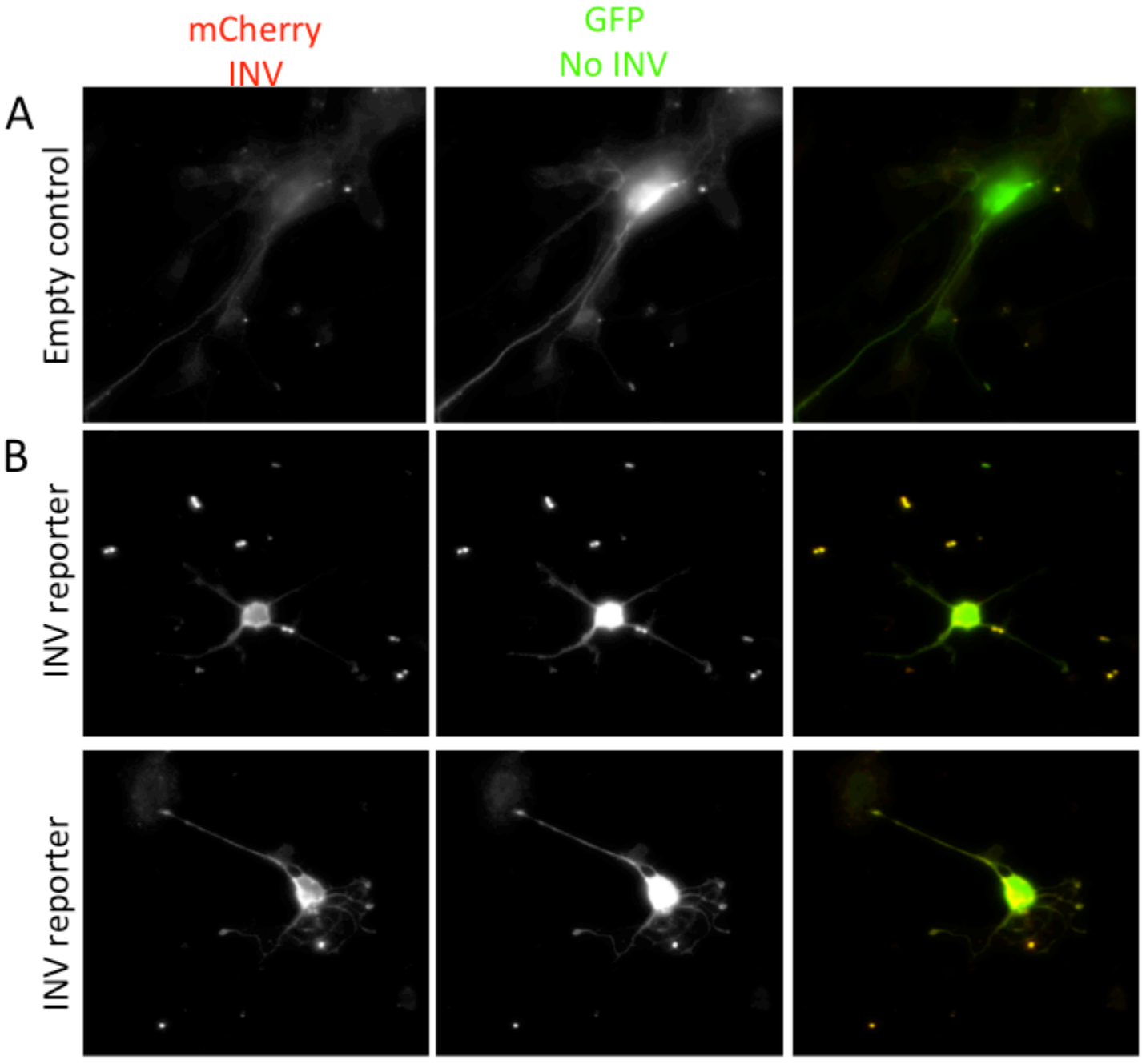

Figure 10: Mena^{INV} bichromatic reporter in neurons
Cortical neurons dissected from E18 mouse brains were dissociated, nucleofected with an empty control vector (A) or the INV reporter (B) and plated on laminin and poly-Lysine and left for 2 days in culture. The empty vector control shows little signal in the red channel compared to when the reporter is expressed suggesting the signal seen in red is indicative of INV inclusion.